Botulinum Toxin: Mechanism of Presynaptic Blockade

Abstract. *The mechanism of action of botulinum toxin was analyzed by the use of calcium ionophores and black widow spider venom. Addition of calcium ionophores to nerve-muscle preparations blocked by botulinum toxin did not increase the frequency of miniature end plate potentials. However, the spider venom elicited a barrage of miniature end plate potentials after blockade by botulinum. Electron micrographs of preparations treated with botulinum toxin and then the spider venom revealed clumping of synaptic vesicles at release sites in the otherwise depleted nerve terminals. These findings indicate that the action of botulinum toxin is not due to deficient storage of acetylcholine in vesicles or blockade of calcium entry into nerve terminals. They suggest that the toxin interferes with the acetylcholine release process itself, possibly by blocking exocytosis at the release sites.*

Botulinum toxin (BOT), the most potent biological poison known, causes fatal neuromuscular paralysis in most vertebrates, in nanogram quantities (*1*). Present evidence indicates that BOT acts presynaptically to prevent the release of acetylcholine (ACh) from cholinergic nerve endings (*2*, *3*), but the precise mechanism of its blocking action is not yet known.

According to present concepts of cholinergic transmission, ACh is synthesized in nerve terminals and stored in quantal amounts within synaptic vesicles (*4*). Quanta of ACh are released spontaneously or in response to depolarization of the nerve terminals (*5*). The release process involves entry of calcium into the presynaptic terminals (*6*) and exocytosis of ACh-containing vesicles at specialized release sites (active zones), which are located exclusively opposite postsynaptic folds (*7*). By interfering with any of these steps, BOT could block neuromuscular transmission. Previous studies have indicated that BOT does not prevent impulses from invading nerve terminals (*8*) nor does it inhibit synthesis of ACh by choline acetyltransferase (*3*). In this study three possible mechanisms of action of BOT have been considered: (i) a reduction in the amount of ACh stored in synaptic vesicles, (ii) interference with calcium entry into the nerve terminals, or (iii) failure of exocytosis of synaptic vesicles.

The mechanism of action of BOT was analyzed by the use of two pharmacological probes: calcium ionophores and black widow spider venom (BWSV). On the basis of our results, we suggest that BOT blocks the exocytosis of ACh-containing vesicles at presynaptic release sites.

The test preparation used throughout these experiments was the left phrenic nerve-hemidiaphragm from adult Swiss mice, mounted on a Silastic platform in a muscle bath containing Trowell's T-8 medium (*9*) at room temperature, under an atmosphere of 95 percent O_2 and 5 percent CO_2. Intracellular recordings of postsynaptic potentials were made with conventional glass microelectrodes filled with 2*M* KCl. Preparations used for electron microscopy were processed by the method of Karnovsky (*10*).

The effect of BOT on neuromuscular transmission was studied in 20 hemidiaphragm preparations by adding type A BOT (*11*) to the incubation medium (0.1 μg/ml). Miniature end plate potentials (mepps) were continuously monitored with focal recordings. In 2 to 3 hours, the frequency of mepps decreased to less than 0.01 sec^{-1}, which is below 1 percent of the normal value. The amplitude of the mepps was also reduced, and it became difficult to distinguish the smallest mepps from the baseline of about 60 to 100 μv. These findings are consistent with reports (*8*, *12*) that BOT reduces both the frequency and the amplitude of mepps. After about 5 hours of BOT incubation, neuromuscular transmission could no longer be elicited by supramaximal tetanic nerve stimulation.

In order to determine whether calcium influx could restore synaptic transmission at nerve terminals fully blocked by BOT, the effects of two calcium ionophores (X537A and A23187) were studied. These ionophores are carboxylic antibiotics that produce permeability of membranes to cations including calcium (*13*). The drug X537A has been shown to increase quantal release of ACh at frog neuromuscular junctions in the presence of calcium (*14*). In control experiments, the addition of X537A (25 μM) to mouse hemidiaphragm preparations caused a five- to tenfold increase in mepp frequency within 30 minutes. This increase lasted about 10 to 20 minutes and was followed by a decline to very low frequencies (< 0.01 sec^{-1}). The increase in mepps was dependent on the presence of Ca^{2+} in the bathing medium (Fig. 1A). A similar, but less consistent, increase in mepp frequency was produced by the addition of the ionophore A23187 (25 μM) to the medium. By contrast, BOT-treated preparations did not respond to the ionophores. The addition of X537A or A23187 to the medium after the mepps had been maximally suppressed by BOT failed to elicit any increase in mepp frequency (Fig. 1A). These findings suggest

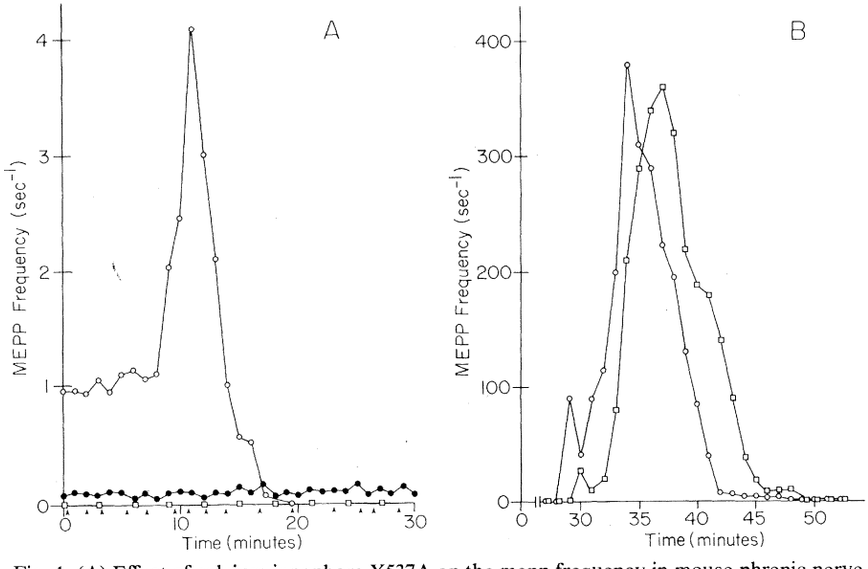

Fig. 1. (A) Effect of calcium ionophore X537A on the mepp frequency in mouse phrenic nerve-diaphragm preparations. Mepps were monitored continuously in an end plate in each experiment. X537A (25 μM) was added to the bath at zero time. (○) Control diaphragm that had been incubated in T-8 medium for 5 hours. (□) Diaphragm treated with BOT (0.1 μg/ml) for 5 hours in vitro. Each arrowhead under the time axis indicates the occurrence of a single mepp in the BOT-blocked junction. (●) Control diaphragm incubated in calcium-free bath medium containing 1 m*M* EDTA. The X537A elicited an increase of mepp frequency in the normal preparation in the presence of calcium. There was no increase of mepp frequency in the BOT-treated diaphragm. (B) Effects of BWSV on the mepp frequency of a control neuromuscular junction (○) and one that had been incubated with BOT (□). BWSV was added to the bath at zero time. The two curves are similar.

that Ca^{2+} influx does not reverse the effect of BOT. It is therefore unlikely that BOT acts by interfering with Ca^{2+} entry.

A second pharmacologic agent, BWSV (*15*), was used to analyze the BOT-induced neuromuscular block further; BWSV has been shown to cause calcium-independent release of ACh vesicles from amphibian and mammalian nerve terminals (*16*), and we have used it in our experiment to elicit quantal release from BOT-blocked preparations. When BWSV (one homogenized venom gland per 2 ml) was added to the bath medium of control (nonbotulinized) mouse hemidiaphragm preparations, a massive barrage of mepps was recorded within 10 to 30 minutes. The maximal frequency was usually higher than 300 sec^{-1}. The frequency then quickly declined, and within 20 minutes release ceased completely (Fig. 1B). Prior treatment with BOT did not prevent the action of BWSV. In preparations fully blocked by BOT, the addition of BWSV still produced massive release of ACh, comparable in frequency and time course to that occurring in the nonbotulinized muscles (Fig. 1B). A similar effect has been reported with brown widow spider venom (*17*). This finding indicates that the synaptic vesicles in BOT-treated terminals are not depleted of ACh.

Electron micrographs revealed differences between the nerve terminals treated with BWSV alone and those first blocked with BOT before BWSV treatment; BWSV-treated control preparations showed expanded nerve terminals virtually devoid of vesicles (Fig. 2B), similar to those previously described (*16*). In preparations fully blocked with BOT and then treated to exhaustion with BWSV, expansion and depletion of the nerve terminals were also present; but, in addition, clumping of vesicles at the release sites was commonly observed (Fig. 2C). Of 45 nerve terminals treated with BOT and BWSV, 30 showed clusters of synaptic vesicles at the release sites opposite postjunctional folds. This ultrastructural pattern is not seen in normal mammalian nerve terminals (Fig. 2A) or those blocked with BOT, and was present in only one of 30 terminals treated with BWSV alone. Our electron microscopic observations suggest that BOT interfers in some manner with the process of vesicle release, but the change is visualized only when brought out by the forced exocytosis of ACh vesicles induced by BWSV.

These experimental observations have led us to draw certain conclusions about the mechanism of action of BOT. First, it is unlikely that BOT interferes with storage of ACh in synaptic vesicles. The release of barrages of ACh quanta by BWSV indicates that an abundance of ACh-containing vesicles is present in BOT-blocked terminals. Second, BOT probably does not act by blocking calcium entry into nerve terminals, since Ca ionophores failed to restore quantal release of ACh after BOT blockade. The remaining possibility is that the action of BOT occurs at a stage in the sequence of ACh release after the entry of calcium, possibly by interfering with the exocytosis process itself. This is consistent with our electron micrographs, which show "logjams" of vesicles located mainly at the release sites. On the basis of these observations we propose that

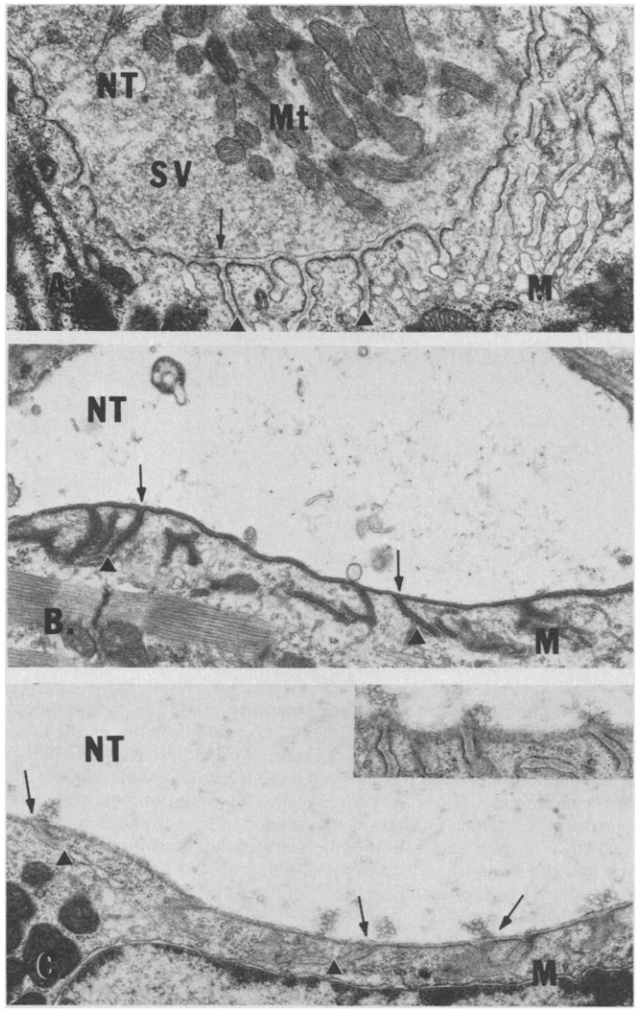

Fig. 2. Electron micrographs of neuromuscular junctions from phrenic nerve-hemidiaphragm preparations. (A) Control neuromuscular junction incubated in Trowell's medium for 6 hours. The nerve terminal (*NT*) contains abundant synaptic vesicles (*SV*) and mitochondria (*Mt*); muscle (*M*); arrows indicate active zone opposite junctional folds (indicated by ▲) (× 19,500). (B) Black widow spider venom (one gland per 2 ml) treatment for 1 hour after incubation in T-8 medium for 5 hours. The nerve terminal (*NT*) is depleted of vesicles. There is no accumulation of vesicles at the active zones (arrows) opposite the junctional folds (▲); muscle (*M*) (× 18,000). (C) Botulinum toxin incubation (0.1 μg/ml) for 5 hours followed by black widow spider venom for 1 hour. The interior of the nerve terminal (*NT*) is similarly depleted of vesicles. Clusters of synaptic vesicles are present at the active zones (arrows) opposite the junctional folds (▲); muscle (*M*) (× 18,000). The insect shows a high-power view of clumping of vesicles at the release sites (× 25,000).

BOT may interact with some membrane component involved in the mechanism of exocytosis in cholinergic nerve terminals.

Note added in proof: Pumplin and Reese (*18*), on the basis of freeze-fracture studies of frog neuromuscular junction treated with BOT, brown widow spider venom, and calcium ionophores, suggest that BOT may block a step in the release of ACh after the stage of entry of cations into nerve terminal.

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Mitochondria: System for Prolonged Maintenance and Repeated Measurements of Energy-Linked Functions

Abstract. *A combination of long-lived mitochondria and a system equipped with gas and liquid exchange hollow fibers permits the maintenance and repeated assay of mitochondria under controlled conditions for periods in excess of 48 hours at room temperature.*

The quasi-autonomous nature of mitochondria reinforces the need to assess their intrinsic metabolic capabilities as expressed within the living cell. Relatively quick and versatile polarographic and spectrophotometric methods of assay have proved valuable in elucidating specific mitochondrial functions. However, extrapolation of these functions to the whole cell requires that they be examined over extended periods of time at or near equilibrium conditions that simulate the homeostatic milieu of a living cell.

Shown schematically in Fig. 1 is a prototype system that permits control over the mitochondrial milieu while allowing repeated assay for oxygen consumption and related energy-dependent functions of the same mitochondria. The essentials of the system are (i) a monitoring chamber equipped with polarographic O_2 electrode, stirrer, and rubber septum; (ii) peristaltic pump; (iii) gas permeator and liquid exchange hollow fibers; and (iv) inert tubing. The gas and chemical constituents of the circulating mitochondrial suspension can be maintained or moderated by manipulating the external gas and liquid medium with which the suspension is in near equilibrium. To measure O_2 consumption, a suitable O_2 partial pressure is established in the mitochondrial suspension, the flow interrupted, and the disappearance of O_2 from the fixed volume chamber determined by the classic polarographic technique (*1*). Adenosine diphosphate (ADP), cofactors, inhibitors, and so forth, may be added through the septum. In practice the flow pump is turned on again before the O_2 is depleted to avoid anoxia. Alternatively, a bypass can be installed parallel to the chamber so that a portion of the mitochondrial suspension is "trapped" for assay while the remainder continues to circulate (*2*).

A continuous recorder trace from a typical series of polarographic assays is shown in Fig. 2. A first and then a second portion of mitochondria were gently injected through the septum into the circulating reaction medium. Circulation was then stopped and ADP added to produce a state 3 oxidation rate. The pump was then turned on and air introduced into the circulating mitochondrial suspension through the gas permeator hollow fiber. Within a few minutes the flow was again stopped and ADP injected in the cham-

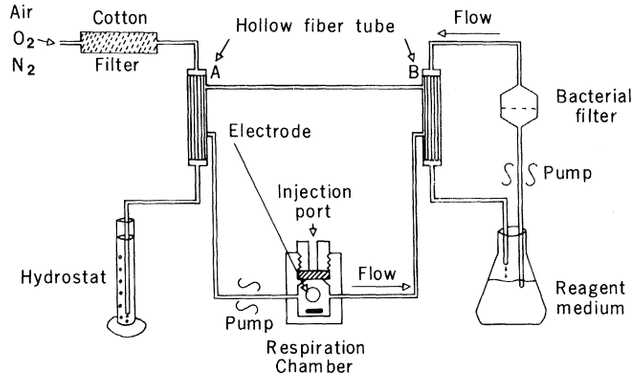

Fig. 1. Prototype mitochondrial maintenance system. Mitochondria suspended in the reagent medium described in the legend to Fig. 3 are circulated (0.5 to 1 ml/min) counterclockwise through the respiration chamber, around the exterior surfaces of liquid exchange (*B*) and gas permeator hollow fibers (*A*), and back to the pump. The mitochondrial suspension is in near equilibrium with a large volume (50 to 100 ml) of complete reaction medium (less mitochondria), which is pumped continuously in counterflow (0.5 to 1 ml/min) through the liquid exchange hollow fibers. A Millipore filter helps prevent occlusion of the liquid exchange hollow fibers (Bio-Rad 80), which have a nominal cutoff for molecules of molecular weight 30,000. The silicone rubber gas permeator membranes (Bio-Rad 5) are essentially nonporous to other than gas molecules. Gas flow through the permeator fibers is maintained at 5 to 10 ml/min against a hydrostatic head of about 20 cm. For more rapid aeration before assay, gas outflow through the permeator can be restricted and the pressure increased up to 8 pounds per square inch. A soft rubber septum at the chamber serves as an injection port. The volume of the closed system, which includes the 1.5-ml chamber, tubing of ⅛-inch inner diameter, and small volume hollow fibers (Bio-Rad π tubes) is approximately 6 ml. A smaller circulating volume but with slower gas exchange rates is achieved by replacing hollow fiber *B* with a dual-function gas and liquid transfer hollow fiber (Bio-Rad 80/5) and incorporating a gas permeator hollow fiber in the external medium flow just before it enters *B*.